Non-Small Cell Lung Cancer Cyclooxygenase-2-dependent Regulation of Cytokine Balance in Lymphocytes and Macrophages: Up-Regulation of Interleukin 10 and Down-Regulation of Interleukin 12 Production

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ABSTRACT

Tumor-derived prostaglandin E₂ (PGE₂) modifies cytokine balance and inhibits host immunity. We hypothesized that a high level of PGE₂ production by lung tumor cells is dependent on tumor cyclooxygenase (COX)-2 expression. We found that PGE₂ production by A549 non-small cell lung cancer (NSCLC) cells was elevated up to 50-fold in response to interleukin (IL)-1β. Reversal of IL-1β-induced PGE₂ production in A549 cells was achieved by specific pharmacological or antisense oligonucleotide inhibition of COX-2 activity or expression. In contrast, specific COX-1 inhibition was not effective. Consistent with these findings, IL-1β-induced COX-2 mRNA expression and protein production in A549 cells. Specific inhibition of COX-2 abrogated the capacity of IL-1β-stimulated A549 cells to induce IL-10 in lymphocytes and macrophages. Furthermore, specific inhibition of A549 COX-2 reversed the tumor-derived PGE₂-dependent inhibition of macrophage IL-12 production when whole blood was cultured in tumor supernatants. Our results indicate that lung tumor-derived PGE₂ plays a pivotal role in promoting lymphocyte and macrophage IL-10 induction while simultaneously inhibiting macrophage IL-12 production. Immunohistochemistry of human NSCLC tissues obtained from lung cancer resection specimens revealed cytoplasmic staining for COX-2 within tumor cells. This is the first description of functional COX-2 expression by NSCLC cells and the definition of a pathway whereby tumor COX-2 expression and a high level of PGE₂ production mediate profound alteration in cytokine balance in the lung cancer microenvironment.

INTRODUCTION

Tumors have the capacity to mediate profound immunosuppression (1–4). In particular, lung tumors produce a variety of mediators that exhibit potent immunosuppressive effects (5–8). In addition to producing their own suppressive factors, tumor cells may also direct surrounding inflammatory cells to release suppressive cytokines in the tumor milieu (8, 9). Tumor-derived PGE₂ is one mediator that orchestrates an imbalance in the production of suppressive and immune-potentiating cytokines by lymphocytes and macrophages in the tumor environment (8).

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PGs³ are produced by the action of cyclooxygenase enzymes on the free arachidonic acid liberated from membrane phospholipids by phospholipases. Prostaglandin endoperoxidase (also referred to as cyclooxygenase or prostaglandin G/H synthase) is the rate-limiting enzyme for the production of PGs and thromboxanes from free arachidonic acid (reviewed in Ref. 10). The enzyme is bifunctional, containing fatty acid cyclooxygenase activity (producing PGE₂ from arachidonic acid) and PG hydroperoxidase activity (converting PGE₂ to PGH₂). Two forms of COX have now been described: a constitutive enzyme (COX-1) present in most cells and tissues; and an inducible isoenzyme (COX-2, also referred to as PGS-2) expressed in response to cytokines, growth factors, and other stimuli (10–15). Our present studies focus on the role of lung tumor COX-2 expression and PGE₂ production in modulating lymphocyte and macrophage-derived cytokines in the tumor microenvironment.

IL-10 and IL-12 are critical regulatory elements of cell-mediated immunity. Whereas IL-10 inhibits cellular immunity, IL-12 induces type 1 cytokine production and effective cell-mediated immunity (16, 17). IL-10 overproduction at the tumor site has been implicated in tumor-mediated immunosuppression (18, 19). In contrast, IL-12 is critical for effective antitumor immunity (20, 21). Tumor models indicate that the tumor-bearing state induces lymphocyte and macrophage IL-10 production but inhibits macrophage IL-12 (22, 23). Thus, whereas IL-12 is the key inducer of type 1 cytokines, IL-10 production at the tumor site may inhibit type 1 cytokine production and cell-mediated antitumor immunity.

In previous studies, we found a 10- to 100-fold increase in human PBL IL-10 production after exposure to NSCLC supernatants (8). The tumor-induced increase in PBL IL-10 production was partially blocked by pretreatment of the tumors with the PG inhibitor indomethacin, and NSCLC lines were found to constitutively produce PGE₂. Multiple PBL subpopulations produced IL-10 in response to exogenous PGE₂ or NSCLC supernatants (8).

Investigations have suggested a role for COX-2 expression in promoting colon tumor invasion and metastases, but the immunological significance of COX-2 expression by tumors has not been defined (24). We postulated that induction of lung tumor COX-2 by cytokines and growth factors in the tumor environment would lead to high level NSCLC PGE₂ production and subsequent dysregulation of IL-10/IL-12 balance in lymphocytes and macrophages. Although recent studies have indicated that COX-2 is expressed in colon, gastric, and breast carcinomas (25–27), COX-2 expression in NSCLC and its role in immune modulation have not been described previously. In this study we report that: (a) COX-2 is expressed in NSCLC in response to IL-1β and is the dominant pathway responsible for a high level of PGE₂ production; (b) elevated PGE₂ production by NSCLC cells leads to up-regulation of lymphocyte and macrophage IL-10 and IL-12.

³ The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; IL, interleukin; NSCLC, non-small cell lung cancer; EIA, enzyme immunoassay; PBL, peripheral blood lymphocyte; PBS, fetal bovine serum; mAb, monoclonal antibody.
down-regulation of macrophage IL-12 production; and (c) abrogation of tumor COX-2 production reverses both (a) and (b). In addition, because it has not been determined previously that COX-2 is present in human NSCLC in situ, we evaluated NSCLC tissues obtained from operative resection specimens and have documented tumor cell COX-2 staining by immunohistochemistry. This is the first report of functional COX-2 expression in human lung cancer and the first documentation of tumor COX-2-dependent regulation of cytokine balance in lymphocytes and macrophages. Our findings suggest that COX-2 expression by lung tumor cells may be pivotal in regulating cytokine balance at the tumor site.

MATERIALS AND METHODS

Reagents. PGE\textsubscript{2}, dexamethasone, and aspirin were obtained from Sigma Chemical Co. (St. Louis, MO). NS398 was obtained from Cayman Chemical Company (Ann Arbor, MI). Human recombinant IL-1β (specific activity, 280 units/mg) was obtained from Genzyme (Cambridge, MA). Specific anti-COX-1 and anti-COX-2 antibodies were obtained from Oxford Biomedical Research, Inc. (Oxford, MI).

Cell Culture. The human lung adenocarcinoma cell line, A549, was obtained from American Type Culture Collection (Rockville, MD). SKLU-1 is a lung adenocarcinoma line provided by Dr. J. A. Radosevich. The human lung squamous cell carcinoma cell line RH2 was established in our laboratory from a surgical resection specimen. The cells were grown in 5% CO\textsubscript{2} in air as monolayers at 37°C in 25-cm\textsuperscript{2} tissue culture flasks containing 5.0 ml of RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine (JRH Biosciences, Lenexa, KS).

Preparation of Human Peripheral Blood Lymphocytes and Macrophages. Human PBLs were prepared from normal healthy donors as described previously (8). Briefly, mononuclear cells were separated by Ficoll-HyPaque gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ). After two washes with PBS, mononuclear cells were cultured in RPMI 1640 supplemented with 10% FBS, 5% human AB serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 81.4 µg/ml MEC nonessential amino acids. After incubation at 37°C for 3 h, the nonadherent lymphocyte population was separated from the adherent macrophage population. PBLs were collected and adjusted to a concentration of 2 × 10\textsuperscript{5} cells/ml in RPMI 1640 containing 2% human AB serum (complete medium) or in A549-conditioned medium (24-h culture supernatant) for further studies.

Whole Blood Culture. Human whole blood was obtained from healthy donors. A549 cells (10\textsuperscript{6}) were cultured in 24-well tissue culture plates in the following conditions: medium alone, NS398 (1 µM), IL-1β (280 units/ml), or IL-1β plus NS398 for 24 h. Complete medium or A549 supernatants from different conditions were then collected and added to the whole blood from healthy donors (9:1, tumor supernatant:whole blood). After 24 h incubation, whole blood IL-12 production was determined by ELISA. PGE\textsubscript{2}, EIA. The PGE\textsubscript{2} monoclonal EIA kit was obtained from Cayman Chemical Company (Ann Arbor, MI). PGE\textsubscript{2} EIA was performed according to the manufacturer's instructions. Briefly, PGE\textsubscript{2}-acetylcholinesterase conjugate, mouse anti-PGE\textsubscript{2} monoclonal antibody, and either standard or sample were added to each well of an EIA plate precoated with goat anti-mouse antibody and blocked with blocking proteins. After 18 h incubation at 25°C, the plate was washed five times to remove unbound reagents. Eillman's reagent was then added to each well, and the plate was read at 405 nm with a plate reader (Dynatech, Chantilly, VA). The results are expressed as ng/ml per 10\textsuperscript{6} cells per 24 h and are representative of at least three experiments performed in triplicate.

Preparation of COX-1, COX-2, and β-Actin Plasmid cDNA Probes. Human COX-1 and COX-2 plasmid cDNA probes were described previously (13). The human β-actin plasmid cDNA probe was obtained from American Type Culture Collection. The human COX-1 plasmid cDNA insert (1.8-kb HindIII/XbaI fragment), human COX-2 plasmid cDNA insert (1.8-kb EcoRI/ApaI fragment), and human β-actin plasmid cDNA insert (1.9-kb EcoRI fragment) were purified by the UltraClean method (Mo Bio Labs, Inc. Solana Beach, CA). The purified human COX-1, COX-2, and β-actin inserts were labeled with [\textsuperscript{32}P]dCTP by nick translation. The concentration of the probes used in hybridization was 10\textsuperscript{6} cpm/nl.

RNA Blot Analysis. Total RNA isolation and Northern blot analysis were performed as described previously (8). Briefly, 10\textsuperscript{7} A549 cells from each condition were lysed in 2 ml of guanidine thiocyanate (4 M)/2-mercaptoethanol (1 mM) solution by gentle pipetting. Two ml of acidified phenol and 200 µl of chloroform were added to the cell lysate and mixed. The cell lysate mixture was centrifuged at 12,000 × g for 10 min at 4°C, and the upper aqueous phase was collected. Equal volumes of isopropanol were added to the upper aqueous phase and kept at −20°C for 1 h. After centrifugation at 12,000 × g for 10 min at 4°C, the RNA pellet was washed twice with 75% ethanol and dried briefly under vacuum. The dried RNA pellet was dissolved in 0.1% diethyl pyrocarbonate-treated, double-distilled H\textsubscript{2}O. For Northern blot analysis, 20 µg of total RNA from each sample were subjected to 1% formaldehyde-denatured agarose gel electrophoresis. The gel was photographed and transferred to a nylon membrane. The RNA was immobilized on the membrane by a UV cross-linker (Stratagene, La Jolla, CA). The filters were prehybridized and hybridized with a 32P-labeled human COX-1, human COX-2, or β-actin plasmid cDNA probe. After washing, the filters were exposed to Fuji medical X-ray films (Fisher Scientific, Santa Clara, CA). The relative intensity of COX-1 and COX-2 mRNA versus β-actin mRNA was quantified using an Alphalmager 2000 documentation and analysis system (Alpha Innotech Corp., San Leandro, CA).

Determination of COX-2 Protein Synthesis by Immunoprecipitation. The determination of COX-2 protein synthesis was performed as described previously (28). Briefly, 3 × 10\textsuperscript{5} A549 cells were plated in a 25-cm\textsuperscript{2} tissue culture flask and maintained according to routine culture conditions. After 24 h incubation, the medium from each culture was discarded, and the cell monolayers were washed three times with PBS and replaced with RPMI 1640 containing 2% FBS with or without IL-1β (280 units/ml). At different time points, the culture medium was replaced with methionine-free medium containing 1\textsuperscript{35}S-methionine (200 µCi/ml; NEN Research Products, Wilmington, DE) and incubated for 30 min. The radioactive medium was removed, and monolayers were washed twice in PBS and detached by trypsin treatment. Cell pellets were lysed by boiling in 1% SDS (200 µl) for 5 min. After cooling, cell lysate was neutralized in buffer (1000 µl) containing 60 mM Tris-Cl (pH 7.4), 190 mM NaCl, 6 mM EDTA, and 2.5% Triton X-100. Excess anti-COX-2 antibody was added to the cell lysate and incubated at 4°C for 2 h with continuous rotation. Protein A-Sepharose CL-4B (50 µl; Sigma) in 1:1 dilution with double-distilled H\textsubscript{2}O was added and incubated at 4°C for 2 h. The samples were pelleted by centrifugation at 12,000 × g for 1 min and washed twice in buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, and 0.02% SDS, followed by three washes in the same buffer without Triton and SDS. The COX-2/anti-COX-2 complexes adsorbed to protein A-Sepharose from each sample were released by boiling for 5 min in 50 µl of buffer containing 60 mM Tris-Cl (pH 6.8), 2% SDS, 2% mercaptoethanol, 0.01% bromphenol, and 10% glycerol before PAGE. The gel was then dried and exposed to Fuji medical X-ray films. The relative intensity of the M\textsubscript{r} 71,000 COX-2 protein band in IL-1β-treated samples versus controls was quantified using the Alphalmager 2000 documentation and analysis system.

Western Blot Analysis. Cell lysates from the three NSCLC cell lines A549, SKLU-1, and RH2, with or without IL-1β (280 units/ml) treatment, were applied to PAGE. The gel was transferred to a nitrocellulose membrane using Mini Trans-Blot electrophoretic transfer cell (BIO-RAD Laboratories, Hercules, CA). ECL Western blotting kit was obtained from Amersham Corp. (Arlington Heights, IL). ECL detection of COX-2 protein production was performed according to the manufacturer's instructions. The relative intensity of the M\textsubscript{r} 71,000 COX-2 protein band in IL-1β-treated samples versus control was quantified using the Alphalmager 2000 documentation and analysis system.

COX-2 Phosphorothioate Antisense Oligonucleotide Studies. Human COX-1 phosphorothioate antisense oligonucleotides (5'-CCG GAG CAA GAG CCG ACT CCT GAT CCA GAG ACG ACG TTC TTT TAG TAG TAC-3'), and their sense counterparts were synthesized by Bio-Synthesis, Inc. (Lewisville, TX). The COX-1 and COX-2 antisense oligonucleotides are complementary to the sequence beginning six nucleotides preceding the initiation codon of the COX-1 and COX-2 mRNAs, respectively. Briefly, A549 cells were cultured to 50% confluence. COX-1 or COX-2 phosphorothioate antisense oligonucleotides (5 µM) were then added to the wells 18 h before the addition of IL-1β (280 units/ml). COX-1 or COX-2 phosphorothioate sense...
oligonucleotides were used as controls. After an 18-h incubation, the supernatants were collected for PGE₂ determination by EIA.

Cyclooxygenase Inhibition and Anti-PGE₂ Antibody Studies. A549 cultures were pretreated with a selective COX-2 inhibitor, NS398 (1 μM; Ref. 29), dexamethasone (20 μM, which suppresses induced COX-2 gene expression but not COX-1 gene expression), with specific anti-PGE₂ mAb (15.7 μg/ml, clone 2B5; Ref. 30) or control antibody (15 μg/ml, mouse IgG₁ isotype control) for 2 h before the addition of IL-1β (280 units/ml). Both anti-PGE₂ and control mAb were generously provided by Dr. Joseph Portanova (G. D. Searle). Aspirin pretreatment was used for specific COX-1 inhibition. The medium from cultures pretreated with aspirin (50 μM) was discarded after 2 h incubation, the monolayers were washed five times with PBS, and fresh medium containing IL-1β (280 units/ml) was added. At different time points, the A549 supernatants were collected for the determination of PGE₂ concentration by EIA.

ELISA. Rat anti-human IL-10 capture monoclonal antibody and rat biotinylated anti-human IL-10 monoclonal antibody pairs were obtained from PharMingen (San Diego, CA). ELISA was performed as described above, except that plates were coated with rat anti-human capture monoclonal antibody and biotinylated anti-human IL-10 monoclonal antibody was used as the detection antibody. The human IL-12 ELISA kit was obtained from R&D Systems (Minneapolis, MN), and the assay was performed according to the manufacturer’s instructions. This ELISA kit uses a capture antibody that recognizes only the IL-12 heterodimer and not the individual p35 and p40 subunits.

Immunohistochemistry. Human normal lung tissues and lung tumor nodules were obtained from the West Los Veterans Administration Medical Center Anatomical Pathology Laboratory in paraffin-embedded blocks. Fifteen tumors (8 adenocarcinomas and 7 squamous carcinomas) and 15 normal lung specimens were evaluated by immunohistochemistry. Four-μm tissue sections were affixed to microscope slides and deparaffinized. The slides were first incubated in a microwave oven in 0.1 M citrate buffer, pH 6.0 (31). Slides were then immersed in 1.5% hydrogen peroxide/methanol for 15 min and then in normal goat serum (1.5%) for 30 min to block endogenous peroxidase activity and nonspecific binding sites, respectively. Immunostaining was performed with a rabbit polyclonal IgG specific for human COX-2 (Cayman Chemical Co.) in a 1:100 dilution at room temperature for 1 h. Sections were treated with biotinylated secondary antibodies and avidin-biotin peroxidase complex solution according to the ABC Quick Kit protocol (Vectastain; Vector Laboratories, Burlingame, CA). The 3,3’diaminobenzidine treatment was also performed as described by the manufacturer (DAB kit, Vectastain; Vector Laboratories). Sections were counterstained with hematoxylin.

Statistical Analysis. Differences between experimental versus control values were evaluated by Student’s t test.

Results

IL-1β Induces A549 PGE₂ Production in a Dose- and Time-dependent Matter. We reported previously that an indomethacin-sensitive component of NSCLC supernatants potently induces PBL IL-10 production (8). NSCLC cell lines maintained in our laboratory constitutively produce PGE₂ in a range of 20–1900 pg/ml per 10⁶ cells per 24 h (8). Thus, we speculated that a portion of the tumor cell capacity of the supernatant capacity to induce PBL IL-10 production is due to tumor-derived PGE₂. Furthermore, we hypothesized that induction of COX-2 and a high level of PGE₂ production by lung tumor cells would lead to enhanced production of IL-10 from lymphocytes and macrophages.

In the present study, IL-1β was found to consistently and potently increase PGE₂ production by A549 tumor cells. To determine the optimal IL-1β concentration for PGE₂ induction from A549, tumor cells (10⁶/well) were incubated with IL-1β (0–1000 units/ml), and the supernatants were collected for the determination of PGE₂ production by EIA. IL-1β enhanced A549 PGE₂ production in a dose-dependent manner. Significant induction of A549 PGE₂ was seen after stimulation with 30 units/ml of IL-1β, and maximal induction occurred with 125–500 units/ml (Fig. 1A). Using these experimental conditions, in multiple replicates of these studies, IL-1β (125–500 units/ml) led to peak A549 PGE₂ production in a range of 10–30 ng/ml. To study the kinetics of PGE₂ induction by IL-1β, A549 cells were incubated with or without the cytokine (280 units/ml), and at different time points (0–36 h), the culture supernatants were assessed for PGE₂ determination by EIA. A549 cells (10⁶) were cultured in complete medium with or without IL-1β (280 units/ml). At different time points (0–36 h), the tumor cell supernatants were collected for PGE₂ determination by EIA. IL-1β induces PGE₂ production in a dose- and time-dependent manner. *, P < 0.05; **, P < 0.01. The PGE₂ EIA results are expressed as ng/ml per 10⁶ cells per 24 h and are representative of four experiments performed in triplicate; bars, SE.
11β Increases Tumor COX-2 mRNA Expression and Protein Synthesis. To determine whether IL-1β-induced PGE₂ production was associated with up-regulation of COX-2, COX-2 mRNA, and protein levels in A549 cells were determined before and after IL-1β treatment. The constitutive COX-2 mRNA expression and protein synthesis were compared with levels after IL-1β induction. A549 cells (10⁷) were cultured in complete medium with IL-1β (280 units/ml). At different time points (0–8 h), the cells were harvested for total RNA isolation and Northern blot analysis. COX-2 mRNA expression was increased 8-fold as early as 1 h in response to IL-1β. In contrast, COX-1 mRNA expression remained unchanged (Fig. 2A). COX-2 expression declined at 2 h but had a second peak starting at the 4-h time point. This later increase may be secondary to a tumor-derived PGE₂-mediated induction of COX-2 mRNA expression (15).

To determine the induction of COX-2 protein in response to IL-1β, immunoprecipitation and Western blot analysis were performed. A549 cells were cultured in complete medium with IL-1β (280 units/ml). At different time points (0–24 h), the cells were lysed for total protein determination. Constitutive and IL-1β-induced COX-2 protein synthesis in A549 was determined by isotope labeling and immunoprecipitation with specific anti-COX-2 antibodies. An increased COX-2 protein synthesis rate in response to IL-1β incubation in A549 cells was demonstrated by increased intensity (3–6-fold increase) of a Mr 71,000 band corresponding to the COX-2 peptide subunit (Fig. 2B). Because no previous studies have documented COX-2 expression and function in NSCLC, we evaluated two additional NSCLC lines for COX-2 protein production in response to IL-1β. As shown in Fig. 2C by Western blot analysis, all three lines showed increased COX-2 protein production (4–11-fold increase) in response to IL-1β. The doublet evident after IL-1β stimulation of A549 (Fig. 2C, Lane 2) is consistent with SDS-PAGE in previous reports in other cell types (33, 34). This has been attributed to partial glycosylation of an N-glycosylation site in the 18-amino acid sequence near the COOH terminus (35). Up-regulation of COX-2 protein synthesis was also demonstrated by immunostaining of IL-1β-treated A549 cells with specific anti-COX-2 antibody (data not shown).

Both COX-2-specific Antisense Phosphorothioate Oligonucleotides and COX-2-specific Pharmacological Inhibitors Abrogate NSCLC-derived PGE₂ Production. To evaluate the contribution of tumor COX-2 in the induction of PGE₂, A549 cells (10⁷) were cultured in complete medium with or without IL-1β (280 units/ml) and either COX-1 or COX-2 sense or antisense oligonucleotides. After a 24-h incubation, the culture supernatants were evaluated for PGE₂ production by EIA. COX-2 antisense oligonucleotides (5 μM), but not COX-1 antisense oligonucleotides (5 μM), completely blocked IL-1β-induced PGE₂ production in A549 cells (P < 0.01; Fig. 3A). Control COX-1 and COX-2 sense oligonucleotides had no effect on the production of PGE₂. These results indicate that a high level of production of PGE₂ by A549 lung tumor cells is COX-2 dependent.

Further confirmation of the importance of COX-2 in the induction of A549 PGE₂ production was obtained through the use of agents that specifically inhibit cyclooxygenase isoenzymes. A549 cells were cultured in the following conditions: medium alone; IL-1β (280 units/ml); IL-1β plus the COX-2-specific inhibitor, NS398 (1 μM); IL-1β plus dexamethasone (20 μM); or IL-1β plus aspirin (50 μM). A549 cells were treated with inhibitors for 2 h before the addition of IL-1β. At different time points (0–24 h), the culture supernatants were collected for the determination of PGE₂ production by EIA. Aspirin was used to assess the pharmacological abrogation of COX-1 in this model. Aspirin has been shown to inhibit both COX-1 and COX-2 isoenzymes irreversibly by covalent modification (35). However, when aspirin is removed from the medium, newly synthesized, IL-1β-induced COX-2 is unaffected and, therefore, active (36). A549 cells were treated with aspirin for 2 h, and the aspirin-containing culture supernatants were discarded. After washing the cell monolayers thoroughly with PBS to remove residual aspirin, fresh medium

Fig. 2. IL-1β increases A549 COX-2 mRNA expression and protein synthesis. A, Northern blot analysis. The filters were hybridized with either COX-1, COX-2, or β-actin plasmid cDNA probes shown in the upper, middle, and lower panels, respectively. COX-2 mRNA expression was increased as early as 1 h in response to IL-1β, whereas COX-1 mRNA expression remained constant. B, immunoprecipitation. [35S]Methionine-labeled COX-2 protein corresponds to a M, 71,000 peptide band separated by PAGE. Increased COX-2 protein synthesis in A549 cells was demonstrated in response to IL-1β. C, Western blot analysis. In response to IL-1β, increased COX-2 protein is seen in all three NSCLC cell lines tested. Lane 1, A549; Lane 2, A549 plus IL-1β; Lane 3, SKLU-1; Lane 4, SKLU-1 plus IL-1β; Lane 5, RH2; Lane 6, RH2 plus IL-1β.
Specific Inhibition of Tumor COX-2 Abrogates the Capacity of A549 to Induce IL-10 in Lymphocytes and Macrophages. We reported previously that both PGE$_2$ and an indomethacin-sensitive component of NSCLC supernatants are potent inducers of PBL IL-10 production (8). Based on the studies described above, we postulated that increased PGE$_2$ production from IL-1ß-stimulated tumors would further enhance the capacity for tumors to induce IL-10 from PBLs and macrophages. We also anticipated that abrogation of either tumor COX-2 synthesis or tumor supernatant PGE$_2$ activity would interrupt the tumor-induced increase of IL-10 production in lymphocytes and macrophages. A549 cells were cultured with or without IL-1ß (280 units/ml) in the presence of specific anti-PGE$_2$ mAb (15.7 mg/ml), control antibody (15.7 µg/ml), or NS398 (1 µM). After a 24-h incubation, A549 supernatants were then collected and added to freshly isolated PBLs (2 X 10$^6$/ml) or macrophages (5 X 10$^5$/ml). After a 24-h exposure to tumor supernatants, PBL and macrophage IL-10 production were determined by ELISA. Specific inhibition of tumor-derived PGE$_2$ activity with anti-PGE$_2$ mAb, or tumor COX-2 expression with NS398, abrogated the IL-10 induction capacity of IL-1ß-treated A549 tumor supernatant (Fig. 4; P < 0.01).

Inhibition of Tumor COX-2 or Abrogation of Tumor Supernatant PGE$_2$ Activity Reverses Tumor-induced Suppression of Macrophage IL-12. PGE$_2$ has been reported to potently inhibit macrophage IL-12 production in an IL-10-independent fashion (37). A decrement in IL-12 production has also been noted in tumor-bearing mice (22). Based on these previous studies, we postulated that tumor-derived PGE$_2$ would act to decrease monocyte/macrophage IL-12 production. IL-12, the predominant macrophage-derived cytokine responsible for induction of the type 1 cytokine pattern, was assessed in whole blood cultures, as described previously by Van der Pouw Kraan et al. (37). After incubation with A549 supernatants, IL-12 production in whole blood cultures was evaluated. A549 tumor supernatant caused a significant decrease in IL-12 production from whole blood cultures (P < 0.01; Fig. 5A). An even more marked inhibition of whole blood IL-12 production was demonstrated when IL-1ß-treated A549 supernatants were added to whole blood cultures (P < 0.01; Fig. 5A). This inhibition could be reversed by specific inhibition of tumor COX-2 with NS398 (Fig. 5A).

To determine whether PGE$_2$ was the COX-2 metabolite responsible for IL-12 inhibition, we assessed whole blood IL-12 production in the presence of specific anti-PGE$_2$ mAb or control antibody. A549 cells were cultured for 24 h with or without IL-1ß (280 units/ml), with control antibody (15.7 µg/ml), or specific anti-PGE$_2$ mAb (15.7 µg/ml). A549 tumor supernatants were then collected and added to whole blood from healthy donors. After a 24-h incubation, whole blood IL-12 production was determined by ELISA. Anti-PGE$_2$ mAb, but not control antibody, reversed the tumor-mediated suppression of IL-12 production in human whole blood cultures (P < 0.01; Fig. 5B).

Immunohistochemical Staining Reveals Localization of COX-2 to Lung Tumor Cells. Because tumor cell lines may differ from tumor cells in situ, we assessed COX-2 staining in NSCLC tissue sections obtained from surgical specimens. Antibodies specific for human COX-2 were used to evaluate NSCLC and normal adjacent lung resection specimens by immunohistochemistry. All of the 15 tumor specimens (8 adenocarcinomas and 7 squamous cell carcinomas) showed cytoplasmic staining for COX-2 in tumor cells (Fig. 6). In contrast, adjacent normal lung showed no COX-2 staining in the normal alveolar lining epithelium but demonstrated positive cytoplasmic staining, often in alveolar macrophages and occasionally in bronchiolar epithelium.
that both tumor-derived and exogenous PGE₂ are potent inducers of IL-10 production by human PBL and monocytes. In addition to inducing IL-10, PGE₂ inhibits macrophage IL-12 production in an IL-10-independent manner (37). Our findings in the present study suggest that PGE₂-induced immunosuppression in NSCLC occurs through interruption of the cytokine balance that would promote cell-mediated antitumor responses. Specifically, tumor-derived PGE₂ both enhances IL-10 production in lymphocytes and macrophages and inhibits macrophage production of IL-12.

We found that a key early regulatory step in this immunosuppressive network is the induction of tumor COX-2 expression, leading to a high level of PGE₂ production by NSCLC cells. This is the first report of functional COX-2 expression in human lung cancer and the first documentation of tumor COX-2-dependent regulation of cyto-

**DISCUSSION**

PGE₂ has long been observed to play a role in tumor-associated immune suppression, but the precise pathways whereby PGE₂ mediates these effects have not been clearly delineated (38). Cyclooxygenase inhibitors have been used to attenuate these effects of PGE₂ in both preclinical models and clinical studies (39, 40). The role of PGE₂ in experimental lung cancer models has also been studied extensively (41–43). Both lung tumors and tumor-associated macrophages release PGE₂ into the tumor environment (44, 45), and inhibition of prostaglandins has been shown to augment antitumor responses (39, 40, 46). Strassmann et al. (47) found that PGE₂ is a potent inducer of murine macrophage IL-10 secretion. Similarly, our previous work indicates...
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Fig. 6. Representative immunohistochemistry staining for COX-2 in NSCLC sections and normal lung. A. adenocarcinoma, X100; B. squamous cell carcinoma, X40; C. normal lung section, X40. Immunoreactive COX-2 is localized to tumor cell cytoplasm but is absent in normal alveolar epithelium.

expression and PGE₂ production. Serum (14, 48), forskolin (49), lipopolysaccharide (50), phorbol esters (51, 52), epidermal growth factor (53), platelet-derived growth factor (54), transforming growth factor β1 (15, 32, 55-57), PGE₂, and other mediators (10) have been shown to regulate COX-2. IL-1β, an important cytokine in pulmonary inflammatory responses (58), has been the cytokine most widely studied in regulation of COX-2 expression. Investigations in a variety of cell types have shown that IL-1β up-regulates COX-2 but has little or no effect on COX-1 expression (11, 14, 57, 59). We found a time- and concentration-dependent induction of COX-2 expression and PGE₂ production by IL-1β in NSCLC cells. Tumor COX-2 expression was found to be crucial to this induction, as evidenced by specific abrogation of its synthesis by antisense oligonucleotides and dexamethasone, or its activity by a pharmacological agent. The rapid and transient induction of COX-2 has been found in other cell types, and this isoenzyme has been characterized as an “immediate early response” gene (10). In contrast to COX-1, COX-2 mRNA expression is relatively unstable, consistent with the presence of multiple RNA instability sequences (AUUUA) in the 3’ untranslated region (35).

Our present results are consistent with our earlier findings: an indomethacin-sensitive component of tumor supernatants induced PBL IL-10 production (8). In the present investigation, antibody blocking studies identified tumor-derived PGE₂ as the key soluble factor responsible for up-regulation of lymphocyte and macrophage IL-10 and inhibition of whole blood IL-12 production. IL-10 inhibits a broad array of immune parameters that include antigen presentation (60), antigen-specific T-cell proliferation (61, 62), and type 1 cytokine production (63, 64). Pretreatment of tumor targets with IL-10 renders the tumor cells more resistant to CTL-mediated lysis (65, 66). We have found previously that production of IL-10 by cutaneous carcinomas provides a mechanism for evasion of the local T-cell immune response (18). We also found that transgenic mice overexpressing IL-10 under the control of the IL-2 promoter were unable to limit the growth of immunogenic tumors (67). Administration of blocking IL-10 mAbs restored in vivo antitumor responses in these transgenic mice. These findings support the suggestion that enhanced lymphocyte-derived IL-10 production antagonizes antitumor immunity (68). In contrast, IL-12 induces Th1 cytokine production and is necessary for effective host cell-mediated antitumor immune responses (17, 69, 70). Macrophage IL-12 production is down-regulated in mice bearing mammary carcinomas (22), and administration of this cytokine has therapeutic antitumor efficacy (71-74). The present results indicate that lung tumor COX-2 expression is responsible for the induction of lymphocyte and macrophage IL-10 as well as the inhibition of whole blood macrophage IL-12 production. Abrogation of tumor-derived PGE₂ restores cytokine balance in our tumor supernatant/lymphocyte-macrophage coculture system. Our results are consistent with the recent findings of Hilkens et al. (75), who found that T-cell IFN-γ production is largely determined by the ratio of IL-12 and PGE₂ at the time of T-cell activation. Thus, a tumor-induced imbalance in the production of either IL-12 or PGE₂ may, therefore, lead to immune dysfunction (22, 75).

Frequent aspirin users have been found to have a decreased occurrence and lower mortality from colon cancer (76). It has been suggested that this beneficial effect of aspirin, which is known to inhibit both COX-1 and COX-2 in a variety of cells (35), may be due in part to inhibition of tumor cyclooxygenase (77). COX-2 is overexpressed in colon carcinomas relative to normal colonic mucosa, and overexpression of COX-2 has been identified as an early central event in colon carcinogenesis (25, 78–81). When overexpressed in a normal epithelial cell line, COX-2 induced a more malignant phenotype and resistance to apoptotic cell death (80). Overexpression of COX-2 in colon tumor lines also enhances tumor invasiveness and thus may also
increase metastatic potential (24). Genetic analysis, using COX-2 knock-out mice, has lent further support to the proposal that the COX-2 gene is a modifying gene for intestinal cancer (82). Thus, in addition to promoting a malignant phenotype, our findings suggest that COX-2 also plays an important role in antagonizing host immune surveillance against malignant cells.

New therapies are needed for NSCLC (5, 83). Despite therapeutic efforts, lung cancer remains the major cause of cancer-related death in the United States (84). Although immunologically based therapies have shown some success for other malignancies, lung cancer has been largely unresponsive. The lung tumor environment is immunosuppressive, and present research focuses on understanding and subverting this problem. Tumor- and inflammatory cell-derived prostaglandins may play an important role in augmenting production of inhibitory cytokines such as IL-10 while suppressing endogenous production of cytokines such as IL-12, which are necessary for effective host cell-mediated antitumor immune responses. Our present findings identify COX-2 in human NSCLC cells in situ and suggest potential new avenues for therapeutic intervention. COX-2 is a focal point of immune-mediated PG production and thus a potential early point of intervention in attempts to restore effective cell-mediated immune responses in the tumor microenvironment. Our present findings add to our understanding of the complex interaction between pulmonary tumor-derived PGE2 and the cytokine network at the tumor site. Tumor COX-2 expression may be an important therapeutic target for pharmacological or gene therapy intervention in NSCLC.

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Non-Small Cell Lung Cancer Cyclooxygenase-2-dependent Regulation of Cytokine Balance in Lymphocytes and Macrophages: Up-Regulation of Interleukin 10 and Down-Regulation of Interleukin 12 Production

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